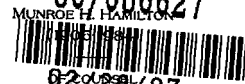


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Sir:

Transmitted herewith for filing is a Continuation-in-Part application of prior  
Serial No. PCT/EP95/03445 filed 9/1/95; which claims priority to  
Danish Application No. 1017/94 filed 9/2/94The teachings of the above applications are incorporated herein by reference in their  
entirety.

Inventor(s): Walter Henry Gunzburg and Robert Michael Saller

Title: NON SELF-INACTIVATING, EXPRESSION TARGETED RETROVIRAL VECTORS

☒ Specification, Claims, Abstract of the Disclosure☒ 17 sheets of ~~XXXXX~~/informal drawings. (Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10,  
11, 12, 13, 14, 15, 16, 17)☐ An assignment of the invention to \_\_\_\_\_☐ A Verified Statement to establish Small Entity Status under 37 C.F.R. 1.9 and  
37 C.F.R. 1.27 is enclosed.☐ A Verified Statement of Small Entity Status was filed in the prior application on  
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Respectfully submitted,  
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APPATENT.LTR-REV. 10/01/96



NON SELF-INACTIVATING, EXPRESSION TARGETED  
RETROVIRAL VECTORS

RELATED APPLICATIONS

This application is a continuation-in-part of  
5 International Application No. PCT/EP95/03445, filed  
September 1, 1995, which claims priority to Danish  
Application No. 1017/94, filed September 2, 1994.

BACKGROUND OF THE INVENTION

The use of retroviral vectors for gene therapy has  
10 received much attention and currently is the method of  
choice for the transferral of therapeutic genes in a  
variety of approved protocols both in the USA and in Europe  
(Kotani et al., Human Gene Therapy 5:19-28 (1994)).  
However most of these protocols require that the infection  
15 of target cells with the retroviral vector carrying the  
therapeutic gene occurs *in vitro*, and successfully infected  
cells are then returned to the affected individual  
(Rosenberg et al., Hum. Gene Ther. 3:75-90 (1992); for a  
review see Anderson, W.F., *Science* 256:808-813 (1992)).  
20 Such *ex vivo* gene therapy protocols are ideal for  
correction of medical conditions in which the target cell  
population can be easily isolated (e.g. lymphocytes).  
Additionally the *ex vivo* infection of target cells allows  
the administration of large quantities of concentrated  
25 virus which can be rigorously safety tested before use.

Unfortunately, only a fraction of the possible  
applications for gene therapy involve target cells that can  
be easily isolated, cultured and then reintroduced.  
Additionally, the complex technology and associated high  
30 costs of *ex vivo* gene therapy effectively preclude its

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disseminated use world-wide. Future facile and cost-effective gene therapy will require an *in vivo* approach in which the viral vector, or cells producing the viral vector, are directly administered to the patient in the form of an injection or simple implantation of retroviral vector producing cells.

This kind of *in vivo* approach, of course, introduces a variety of new problems. First of all, and above all, safety considerations have to be addressed. Virus will be produced, possibly from an implantation of virus producing cells, and there will be no opportunity to precheck the produced virus. It is important to be aware of the finite risk involved in the use of such systems, as well as trying to produce new systems that minimize this risk.

Retroviral vector systems consist of two components (Fig. 1):

1) the retroviral vector itself is a modified retrovirus (vector plasmid) in which the genes encoding for the viral proteins have been replaced by therapeutic genes and marker genes to be transferred to the target cell. Since the replacement of the genes encoding for the viral proteins effectively cripples the virus it must be rescued by the second component in the system which provides the missing viral proteins to the modified retrovirus.

The second component is:

2) a cell line that produces large quantities of the viral proteins, however lacks the ability to produce replication competent virus. This cell line is known as the packaging cell line and consists of a cell line transfected with a second plasmid carrying the genes enabling the modified retroviral vector to be packaged.

To generate the packaged vector, the vector plasmid is transfected into the packaging cell line. Under these conditions the modified retroviral genome including the inserted therapeutic and marker genes is transcribed from the vector plasmid and packaged into the modified retroviral particles (recombinant viral particles). This recombinant virus is then used to infect target cells in which the vector genome and any carried marker or therapeutic genes becomes integrated into the target cell's DNA. A cell infected with such a recombinant viral particle cannot produce new vector virus since no viral proteins are present in these cells. However the DNA of the vector carrying the therapeutic and marker genes is integrated in the cell's DNA and can now be expressed in the infected cell.

The essentially random integration of the proviral form of the retroviral genome into the genome of the infected cell (Varmus, *Science* 240:1427-1435 (1988)) led to the identification of a number of cellular proto-oncogenes by virtue of their insertional activation (Varmus, *Science* 240:1427-1435 (1988); van Lohuizen and Berns, *Biochim. Biophys. Acta*, 1032:213-235 (1990)). The possibility that a similar mechanism may cause cancers in patients treated with retroviral vectors carrying therapeutic genes intended to treat other pre-existent medical conditions has posed a recurring ethical problem. Most researchers would agree that the probability of a replication defective retroviral vector, such as all those currently used, integrating into or near a cellular gene involving in controlling cell proliferation is vanishingly small. However it is generally also assumed that the explosive expansion of a population of replication competent retrovirus from a single infection event, will eventually provide enough integration events to make such a phenotypic integration a very real possibility.

Retroviral vector systems are optimized to minimize the chance of replication competent virus being present. However it has been well documented that recombination events between components of the retroviral vector system can lead to the generation of potentially pathogenic replication competent virus and a number of generations of vector systems have been constructed to minimize this risk of recombination (reviewed in Salmons and Gunzburg, *Human Gene Therapy* 4:129-141 (1993)).

10 A further consideration when considering the use of *in vivo* gene therapy, both from a safety stand point and from a purely practical stand point, is the targeting of retroviral vectors. It is clear that therapeutic genes carried by vectors should not be indiscriminately expressed  
15 in all tissues and cells, but rather only in the requisite target cell. This is especially important if the genes to be transferred are toxin genes aimed at ablating specific tumor cells. Ablation of other, nontarget cells would obviously be very undesirable.

20 A number of retroviral vector systems have been previously describes that should allow targeting of the carried therapeutic genes (Salmons and Gunzburg, *Human Gene Therapy* 4:129-141 (1993)). Most of these approaches involve either limiting the infection event to predefined  
25 cell types or using heterologous promoters to direct expression of linked heterologous therapeutic or marker genes to specific cell types. Heterologous promoters are used which should drive expression of linked genes only in the cell type in which this promoter is normally active.  
30 These promoters have previously been inserted, in combination with the marker or therapeutic gene, in the body of the retroviral vectors, in place of the gag, pol or env genes.

The retroviral Long Terminal Repeat (LTR) flanking  
35 these genes carries the retroviral promoter, which is

generally non-specific in that it can drive expression in many different cell types (Majors, *Curr. Tops. In Micro. Immunol.* 157:49-92 (1990)). Promoter interference between the LTR promoter, and heterologous internal promoters, such as the tissue specific promoters described above has been reported. Additionally, it is known that retroviral LTRs harbor strong enhancers that can, either independently, or in conjunction with the retroviral promoter, influence expression of cellular genes near the site of integration of the retrovirus. This mechanism has been shown to contribute to tumorigenicity in animals (van Lohuizen and Berns *Biochim. Biophys. Acta*, 1032:213-235 (1990)). These two observations have encouraged the development of Self-Inactivating- Vectors (SIN) in which retroviral promoters are functionally inactivated in the target cell (PCT W094/29437). Further modifications of these vectors include the insertion of promoter gene cassettes within the LTR region to create double copy vectors (PCT W089/11539). However, in both these vectors the heterologous promoters inserted either in the body of the vector, or in the LTR region are directly linked to the marker/therapeutic gene.

The previously described SIN vector mentioned above carrying a deleted 3'LTR (PCT W094/29437) utilize in addition a strong heterologous promoter such as that of Cytomegalovirus (CMV), instead of the retroviral 5' LTR promoter (U3-free 5'LTR) to drive expression of the vector construct in the packaging cell line. A heterologous polyadenylation signal is also included in the 3'LTR (PCT W094/29437).

### SUMMARY OF THE INVENTION

The present invention relates to retroviral vectors including a vector which undergoes promoter conversion (ProCon vectors such as, for example, the BAG vector). The

vector system is useful as a gene transfer vehicle for targeted gene therapy.

In particular, the present invention relates to a retroviral vector which is capable of undergoing promoter  
5 conversion comprising a 5' long terminal repeat (LTR) region of the structure U3-R-U5; one or more sequences selected from coding and non-coding sequences; and a 3' LTR region comprising a completely or partially deleted U3 region wherein said deleted U3 region is replaced by a  
10 polylinker sequence, followed by the R and U5 region. The LTR regions can be selected from at least one element of, for example, Murine Leukaemia Virus, Mouse Mammary Tumor Virus, Murine Sarcoma Virus, Simian Immunodeficiency Virus, Human Immunodeficiency Virus, Human T Cell Leukaemia Virus,  
15 Feline Immunodeficiency Virus, Feline Leukaemia Virus, Bovine Leukaemia Virus, and Mason-Pfizer-Monkey Virus.

In one embodiment, the polylinker sequence of the retroviral vector of the present invention comprises at least one unique restriction site. In another embodiment,  
20 the polylinker sequence comprises at least one insertion of a heterologous DNA fragment (e.g., regulatory elements and promoters). In a particular embodiment of the present invention, the regulatory elements and promoters of the heterologous DNA are target cell specific in their  
25 expression. In addition, the regulatory elements and promoters of the retroviral vector can regulate the expression of at least one of the coding sequences of the retroviral vector. The regulatory elements can also be regulatable by transacting molecules. In another  
30 embodiment, the heterologous DNA is DNA homologous to one or more cellular sequences or a part thereof.

The coding sequences of the retroviral vector of the present invention can be, for example, sequences coding for marker genes, therapeutic genes, antiviral genes, antitumor  
35 genes, and cytokine genes. In another embodiment, at least

one of the coding sequences for a retroviral protein (e.g., a retroviral protein involved in integration) is altered or at least partially deleted in the retroviral vector of the present invention.

5       The present invention also relates to a retroviral vector system comprising a retroviral vector which is capable of undergoing promoter conversion comprising a 5' LTR region of the structure U3-R-U5; one or more sequences selected from coding and non-coding sequences; and a 3' LTR  
10 region comprising a completely or partially deleted U3 region wherein said deleted U3 region is replaced by a polylinker comprising regulatory sequences, followed by the R and U5 region; and a packaging cell line (e.g., psi-2, psi-Crypt, psi-AM, GP+E-86, PA317 and GP+envAM-12)  
15 harboring at least one retroviral or recombinant retroviral construct coding for proteins required for said retroviral vector to be packaged. In one embodiment, the packaging cell line harbors retroviral or recombinant retroviral constructs coding for those retroviral proteins which are  
20 not encoded in the retroviral vector.

      The present invention also relates to a method for introducing homologous or heterologous nucleotide sequences (e.g., genes or parts of genes encoding for proteins, regulatory sequences and promoters ) into target human or  
25 animal cell populations *in vitro* and *in vivo* comprising infecting the target cell population with recombinant retroviruses produced by the retroviral vector system of the present invention. In a particular embodiment, introduction of homologous or heterologous nucleotide  
30 sequences into target human or animal cell populations *in vitro* and *in vivo* is accomplished by infecting the target cell population with recombinant retrovirus particles of the present invention.

      The present invention also relates to a recombinant  
35 retroviral particle(s) obtained by transfecting a packaging

cell line of a retroviral vector system of the present invention, and culturing the cells under suitable conditions.

The present invention also relates to a retroviral provirus produced by infection of target cells with a recombinant retroviral particle of the present invention whereby the polylinker in the 3' LTR becomes duplicated during the process of reverse transcription in the target cell and appears in the 5' LTR as well as in the 3' LTR of the resulting provirus.

The present invention also relates to mRNA of the retroviral provirus of the present invention. In addition, the invention relates to RNA of a retroviral vector of the present invention. The present invention also relates to a pharmaceutical composition containing a therapeutically effective amount of a recombinant retroviral particle of the present invention .

#### BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a graphic representation of a retroviral vector system.

Fig. 2 is a graphic representation of reverse transcription of the retroviral genome.

Fig. 3 is a graphic representation of the ProCon principle.

Fig. 4 are the primers used to construct the modified BAG vector.

Fig. 5 is a graphic representation of construction of plasmid pSmaU3del.

Fig. 6 is a graphic representation of construction of plasmid pCON6.

Fig. 7 are the primers used to construct pMMTVgal.

Fig. 8 is a graphic representation of construction of the plasmid pMMTV-BAG.

Fig. 9 are the primers used to construct pWAPBAG.

Fig. 10 is the plasmid pWAPBAG.

Fig. 11 is a graphic representation of a ProCon vector carrying the promoter region from a mouse mammary tumor (MMTV) and the results of PCR analysis of DNA, prepared from clones infected with the vector, using a MLV probe.

Fig. 12 is a graphic representation of a ProCon vector carrying the promoter region from a mouse mammary tumor (MMTV) and the results of PCR analysis of DNA, prepared from clones infected with the vector, using a MMTV probe.

Fig. 13 is a graph of  $\beta$ -galactosidase expression in NIH and EF43 cells infected with MMTV and WAP ProCon vectors.

Fig. 14 is a graph of  $\beta$ -galactosidase expression in primary mammary glands cells from a pregnant mouse infected with MMTV and WAP ProCon vectors.

Fig. 15 is a graph of  $\beta$ -galactosidase expression after virus injection into mammary gland and the skin of a pregnant Balb/c mouse.

Fig. 16 is a graph of  $\beta$ -galactosidase expression in infected mammary tumor cells.

Fig. 17 is a graphic representation of targeted integration of a retroviral vector by homologous recombination.

#### DETAILED DESCRIPTION OF THE INVENTION

The object of the present invention is the construction of a novel retroviral vector which can be used as a safe gene transfer vehicle for targeted gene therapy with a reduced probability to undergo recombination with the packaging construct. This novel vector carries heterologous promoter and/or regulatory elements in the 3'LTR which, after infection become duplicated and translocated to the 5'LTR in the target cell, eventually controlling expression of marker/therapeutic genes, not directly linked to the promoter, but rather inserted into

the body of the vector. This vector does not undergo self-inactivation - but instead promoter exchange, giving rise to the name ProCon for Promoter Conversion.

Since Promoter Conversion does not result in  
5 Self-Inactivation, the retroviral vector will be transcriptionally active in the target cell. However both LTRs will consist to a large extent of heterologous promoter/enhancer sequences in the target cell. This will  
10 reduce the likelihood of the integrated vector in the target cell being subject to the same inactivation over long periods as has been described for conventional vectors (Xu et al., *Virology* 171:331-341 (1989)) and also will  
15 reduce the chance of recombination with endogenous retroviral sequences to generate potentially pathogenic replication competent virus, increasing the safety of the system.

In this invention the 5'LTR of the retroviral vector construct is not modified, and expression of the viral vector in the packaging cells is driven by the normal  
20 retroviral U3 promoter. Normal retroviral polyadenylation is allowed, and no heterologous polyadenylation signals are included in the 3'LTR. This is important for the development of *in vivo* gene therapy strategies, since the normal physiological regulation of the virus, through the  
25 normal viral promoter, and possibly also involving the normal viral control of polyadenylation, will prevail over long periods *in vivo* whilst the packaging cells are producing recombinant virus.

A further modification of this novel retroviral vector  
30 foresees the inclusion of cellular sequences instead of the heterologous promoter and/or regulatory elements. This should allow higher selectivity for site specific recombination with cellular sequences to target the integration of retroviral vectors to particular sites in  
35 the host cell genome (Saller, R.M., "Design von Locus- und

Gewebespezifischen Retroviralen Vektoren Ruer Eine *In Vivo* Gentherapie, Doctoral thesis, Ludwigs-Maximilians University Munich, Germany, (1994)).

To achieve the foregoing and other objects, the  
5 invention provides a retroviral vector undergoing promoter conversion comprising a 5'LTR region of the structure U3-R-US; one or more sequences selected from coding and non-coding sequences; and a 3'LTR region comprising a completely or partially deleted U3 region wherein said  
10 deleted U3 region is replaced by a polylinker sequence, followed by the R and U5 region.

Said polylinker sequence carries at least one unique restriction site and contains preferably at least one insertion of a heterologous DNA fragment. Said  
15 heterologous DNA fragment is preferably selected of regulatory elements and promoters, preferably being target cell specific in their expression, but may also be a DNA fragment with no regulatory function.

Said heterologous DNA fragment is preferably  
20 homologous to one or more cellular sequences. The regulatory elements and promoters are preferably regulatable by transacting molecules.

Further objects, features and advantages will be apparent from the following description of preferred  
25 embodiments of the invention.

The target cell specific regulatory elements and promoters are selected from one or more elements of the group consisting of Whey Acidic Protein (WAP), Mouse Mammary Tumor Virus (MMTV),  $\beta$ -lactoglobulin and casein  
30 specific regulatory elements and promoters, pancreas specific regulatory elements and promoters including carbonic anhydrase II and  $\beta$ -glucokinase regulatory elements and promoters, lymphocyte specific regulatory elements and promoters including immunoglobulin and MMTV lymphocytic  
35 specific regulatory elements and promoters and MMTV

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specific regulatory elements and promoters conferring responsiveness to glucocorticoid hormones or directing expression to the mammary gland. Said regulatory elements and promoters regulate preferably the expression of at least one of the coding sequences of said retroviral vector. The LTR regions are selected from at least one element of the group consisting of LTRs of Murine Leukaemia Virus (MLV), Mouse Mammary Tumor Virus (MMTV), Murine Sarcoma Virus (MSV), Simian Immunodeficiency Virus (SIV), Human Immunodeficiency Virus (HIV), Human T-cell Leukemia Virus (HTLV), Feline Immunodeficiency Virus (FIV), Feline Leukemia Virus (FELV), Bovine Leukemia Virus (BLV) and Mason-Pfizer-Monkey virus (MPMV).

The retroviral vector is based preferably either on a BAG vector (Price et al., *Proc. Natl. Acad. Sci. USA* 84:156-160 (1987)) or an LXS vector (Miller and Rosman, *Biotechniques* 7:980-990 (1989)).

The coding sequence is preferably selected from one or more elements of the group consisting of marker genes, therapeutic genes, antiviral genes, antitumor genes, and cytokine genes.

Said marker and therapeutic genes are preferably selected from one or more elements of the group consisting of  $\beta$ -galactosidase gene, neomycin gene, Herpes Simplex Virus thymidine kinase gene, puromycin gene, cytosine-deaminase gene, hygromycin gene, secreted alkaline phosphatase gene, guanine phosphoribosyl transferase (gpt) gene, alcohol dehydrogenase gene and hypoxanthine phosphoribosyl transferase (HPRT) gene.

Another embodiment of the invention envisages the alteration or partial deletion of at least one retroviral sequence required for integration of retroviruses.

In a further embodiment of the invention a retroviral vector system is provided comprising a retroviral vector as described above as a first component and a packaging cell

line harboring at least one retroviral or recombinant retroviral construct coding for proteins required for said retroviral vector to be packaged.

The packaging cell line harbors retroviral or  
5 recombinant retroviral constructs coding for those retroviral proteins which are not encoded in said retroviral vector. The packaging cell line is preferably selected from an element of the group consisting of psi-2, psi-Crypt, psi-AM, GP+E-86, PA317 and GP+envAM-12, or of  
10 any of these supertransfected with recombinant constructs allowing expression of surface proteins from other enveloped viruses.

Another embodiment of the invention involves the use of a packaging cell line harboring a recombinant retroviral  
15 construct defective in integrase function.

After introducing the retroviral vector of the invention as described above in a retroviral packaging cell line and infection of a target cell, as described above, a retroviral provirus is provided wherein said polylinker and  
20 any sequences inserted in said polylinker in the 3'LTR become duplicated during the process of reverse transcription in the infected target cell and appear in the 5'LTR as well as in the 3'LTR of the resulting provirus.

The invention includes also mRNA of a retroviral  
25 provirus according to the invention and any RNA resulting from a retroviral vector according to the invention.

A further embodiment of the invention provides non-therapeutical method for introducing homologous and/or heterologous nucleotide sequences into human or animal  
30 cells *in vitro* and *in vivo* comprising transfecting a packaging cell line of a retroviral vector system according to the invention with a retroviral vector according to the invention and infecting a target cell population with recombinant retroviruses produced by the packaging cell  
35 line. The nucleotide sequences are selected from one or

more elements of the group consisting of genes or parts of genes encoding for proteins, regulatory sequences and promoters.

5 The retroviral vector, the retroviral vector system and the retroviral provirus as well as RNA thereof is used for producing a pharmaceutical composition for gene therapy in mammals including humans. Furthermore, they are used for targeted integration in homologous cellular sequences.

#### PROMOTER CONVERSION

10 The present invention uses the principle of promoter conversion typical for retroviruses.

The retroviral genome consists of an RNA molecule with the structure R-U5-gag-pol-env-U3-R (Fig. 2). During the process of reverse transcription, the U5 region is  
15 duplicated and placed at the right hand end of the generated DNA molecule, while the U3 region is duplicated and placed at the left hand end of the generated DNA molecule (Fig. 2). The resulting structure U3-R-U5 is called LTR (Long Terminal Repeat) and is thus identical and  
20 repeated at both ends of the DNA structure or provirus (Varmus, *Science* 240:1427-1435 (1988)). The U3 region at the left hand end of the provirus harbors the promoter (see below). This promoter drives the synthesis of an RNA transcript initiating at the boundary between the left hand  
25 U3 and R regions and terminating at the boundary between the right hand R and U5 region (Fig. 2). This RNA is packaged into retroviral particles and transported into the target cell to be infected. In the target cell the RNA genome is again reverse transcribed as described above.

30 According to the invention, a retroviral vector is constructed in which the right-hand U3 region is altered (Fig. 3), but the normal left-hand U3 structure is

maintained (Fig. 3); the vector can be normally transcribed into RNA utilizing the normal retroviral promoter located within the left-hand U3 region (Fig. 3). However the generated RNA will only contain the altered right-hand U3 structure. In the infected target cell, after reverse transcription, this altered U3 structure will be placed at both ends of the retroviral structure (Fig. 3).

If the altered region carries a polylinker (see below) instead of the U3 region then any promoter, including those directing tissue specific expression such as the WAP promoter (see below) can be easily inserted. This promoter will then be utilized exclusively in the target cell for expression of linked genes carried by the retroviral vector. Alternatively or additionally, DNA segments homologous to one or more cellular sequences can be inserted into the polylinker for the purposes of gene targeting, by homologous recombination (see below).

According to the invention, the term "polylinker" is used for a short stretch of artificially synthesized DNA which carries a number of unique restriction sites allowing the easy insertion of any promoter or DNA segment. The term "heterologous" is used for any combination of DNA sequences that is not normally found intimately associated in nature.

Gene expression is regulated by promoters. In the absence of promoter function a gene will not be expressed. The normal MLV retroviral promoter is fairly unselective in that it is active in most cell types (Majors, *Curr. Topics. In Micro. Immunol.* 157:49-92 (1990)). However a number of promoters exist that show activity only in very specific cell types. Such tissue-specific promoters will be the ideal candidates for the regulation of gene expression in retroviral vectors, limiting expression of the therapeutic genes to specific target cells.

In the packaging cell line, the expression of the retroviral vector is regulated by the normal unselective retroviral promoter contained in the U3 region (Fig. 3). However, as soon as the vector enters the target cell

5 promoter conversion occurs, and the therapeutic or marker gene, e.g.,  $\beta$ -galactosidase are expressed from a tissue specific promoter of choice introduced into the polylinker (Fig. 3). Not only can virtually any tissue specific promoter be included in the system, providing for the

10 selective targeting of a wide variety of different cell types, but additionally, following the conversion event, the structure and properties of the retroviral vector no longer resembles that of a virus. This, of course, has extremely important consequences from a safety point of

15 view, since ordinary or state-of-the-art retroviral vectors readily undergo genetic recombination with the retroviral packaging construct and/or endogenous retroviruses to produce potentially pathogenic viruses. Promoter conversion (ProCon) vectors do not resemble retroviruses

20 because they no longer carry U3 retroviral promoters after conversion thus reducing the possibility of genetic recombination.

The retroviral promoter structure is carried within the U3 region of the LTR. LTRs carry signals that allow

25 them to integrate into the genome of the target cell. The integration of retroviral proviruses can also contribute to pathogenic changes (van Lohuizen and Berns, *Biochim. Biophys. Acta*, 1032:213-235 (1990)). In one embodiment of the invention ProCon vectors can carry modified LTRs that

30 no longer carry the signals required for integration. Again this increases the potential safety of these vector systems.



and homologous recombination have been evaluated. Using a cell line carrying a single copy of the HSV-tk gene as a target, we have been able to disrupt the target at frequencies 15 fold higher than previously reported by others (Ellis and Bernstein, *Mol. Cell. Biol.* 9:1621-1627 (1989)). Cloning of the recombined fragments of DNA has revealed the presence of both target tk sequence and retroviral vector (Saller, R.M., "Design von Locus- und Gewebespezifischen Retroviralen Vektoren Ruer Eine *In Vivo* Gentherapie, Doctoral thesis, Ludwigs-Maximilians University Munich, Germany, (1994)).

For targeted integration, DNA segments homologous to cellular sequences are inserted into the polylinker of the ProCon vectors. After infection of the target cell and reverse transcription, these sequences will appear at the 5' terminal end of the provirus. Terminal homologies have been shown to favor homologous recombination (Bradley, *Curr. Opin. Biotechnol* 2:823-829 (1991)) to isogenic cellular sequences (Bradley, *Curr. Opin. Biotechnol* 2:823-829 (1991)). Infection of target cells which carry mutated versions of the homologous sequence should result in the recombination and thus repair of the mutated sequence. Either just the homologous sequences will recombine into the cellular genome, or the complete vector will be inserted (Saller, R.M., "Design von Locus- und Gewebespezifischen Retroviralen Vektoren Ruer Eine *In Vivo* Gentherapie, Doctoral thesis, Ludwigs-Maximilians University Munich, Germany, (1994)). Not only has this vector class potential for use in gene repair, it can also be utilized to direct the integration of retroviral vectors carrying therapeutic genes to specific loci in the genome which are known not to harbor active genes. This will reduce considerably the possibility of insertional activation or inactivation as described above, and will

thus contribute to the safety of the use of retroviral vectors.

The recombinant DNA methods employed in practicing the present invention are standard procedures, well known to those skilled in the art, and described in detail, for example, in "Molecular Cloning" (Sambrook et al., *Molecular Cloning*, Cold Spring Harbor Laboratory Press, New York, USA, (1989) and in "A Practical Guide to Molecular Cloning"; Perbal, B., "A Practical Guide to Molecular Cloning, John Wiley & Sons, 1984)).

The following examples will illustrate the invention further. These examples are however in no way intended to limit the scope of the present invention as obvious modifications will be apparent, and still other modifications and substitutions will be apparent to anyone skilled in the art.

#### EXAMPLE 1

Mammary gland specific expression after infection with ProCon Vectors carrying mammary specific promoters.

#### 20 DELETION OF THE U3 REGION AND INSERTION OF A POLYLINKER

In the murine leukemia virus (MLV) retroviral vector known as BAG (Price et al., *Proc. Natl. Acad. Sci. USA* 84:156-160 (1987))  $\beta$ -galactosidase gene is driven by the promiscuous (i.e., non-tissue specific) MLV promoter in the U3 region of the LTR (Fig. 3). According to the present invention a derivative of the BAG vector has been constructed in which the MLV promoter (U3) located within the 3'LTR, except the inverted repeat, has been deleted by PCR and replaced by a polylinker. The BAG vector lacking the U3 is expressed from the MLV promoter (U3) within the 5'LTR when introduced into a packaging cells line. As a result of the rearrangements occurring the retroviral genome during its life cycle, following infection of its

target cell, the polylinker will be duplicated at both ends of the retroviral genome as described in WO-A1-9607748.

Thereby a retroviral vector can be constructed in which the expression of the  $\beta$ -galactosidase gene of BAG will be

5 controlled by any heterologous promoter inserted into the polylinker.

As a template for PCR we used pBAGN as plasmid carrying a derivative of the BAG construct carrying only one LTR, created by an NheI digest of the original pBAG  
10 followed by a self-ligation of the 7018bp fragment.

The 3' end of primer A is complementary to the R-region of the LTR (Fig. 4). The 5'-extension contains an artificial (art) polylinker and an artificial inverted repeat (IR(art.)). Primer B is complementary to the U5  
15 region of the LTR (Fig. 4). After 35 cycles of annealing at 47°C and extension at 60°C, a 140 bp product was obtained, which was used as a template for the second PCR. In this reaction a Clal site and an artificial (+)PBS was added 5' of the IR-region using primer C (Fig. 4) in  
20 combination with primer B. Annealing was carried out at 53°C and extension at 72°C. After 35 cycles a 163 bp product was obtained, which was digested with Clal and SmaI and ligated to a 2722bp Clal/SmaI fragment of pSmaI (Fig. 5). The resulting plasmid pSmaU3del (2792bp) was  
25 linearized by a SmaI digest and ligated to a 3677bp SmaI fragment of pBAGN (Fig. 6) to give the plasmid pBAGNU3del (6369bp). Deletion of the U3 region was confirmed by sequencing from the Clal-site into the U5-region using pSmaU3del as template. A Clal/Spel (322bp) fragment  
30 containing the U3 deleted LTR was ligated to a Clal/NheI fragment of pBAGNB (created by self ligation of a 3946bp fragment of pBAGN) to give the plasmid pCON6 (4097bp) (Fig. 6). This plasmid carrying a full U3-minus retroviral vector was used as a basis for further cloning.

According to the principle set forth above the following specific promoters have been inserted into the polylinker region or the modified BAG vector: several subregions of the Mouse Mammary Tumor Virus (MMTV) promoter, including a region that confers responsiveness to glucocorticoid hormones and a region containing an element that directs expression to the mammary gland; the Whey Acidic Protein (WAP) promoter. This promoter controls the expression of WAP so that it is only produced in the mammary glands of pregnant and lactating rodents.

#### CLONING OF pMMTVgal

The Mouse Mammary Tumour Virus (MMTV) U3-Region (mtv-2) without the inverted repeats includes a region that confers responsiveness to glucocorticoid hormones and a region containing an element that directs expression to the mammary gland.

The U3 region of MMTV was amplified by PCR using the plasmid pBG102 (a plasmid containing the 3' LTR from mtv 2) as template with primers D and E.

The 3' end of primer D is complementary of the 5' end of MMTV U3 region and carries a SacII site in its 5' extension (Fig. 7). The 3' end of primer E is complementary to the 3' end of the MMTV U3 region and has a MluI site in its 5' extension (Fig. 7).

After 35 cycles of annealing at 49°C and extension at 72°C, a 1229bp product was obtained, digested with SacII and MluI and ligated to the SacII/MluI digested vector pCON6. The resulting plasmid p125.6 (5305bp) (Fig. 8) was digested with XbaI and HindIII and the 4187 bp fragment ligated to the 4190bp fragment of pBAGN containing the  $\beta$ -galactosidase gene to give the plasmid pMMTV-BAG (8377bp) (Fig. 8) in which the  $\beta$ -galactosidase gene is under the

transcriptional control of the MLV promoter after transfection, and under the MMTV promoter after infection.

CLONING OF THE WHEY ACIDIC PROTEIN (WAP) PROMOTER REGION ENCOMPASSING THE PROXIMAL 445 BP OF THE WAP PROMOTER

5 INCLUDING THE TRANSCRIPTION INITIATION SITE

A plasmid, pWAPBAG containing the  $\beta$ -galactosidase gene under transcriptional control of the proximal 445 bp's of the WAP promoter was prepared by amplification of a sequence comprising the proximal 445 bp's of the WAP  
10 promoter and the first 143 bp's of the human growth hormone (HGH). The sequences were amplified from pWAP2-HGH (Günzburg et al., 1991) by PCR using primers F and G (Fig. 9). Both primers carried SacII and MluI recognition sites as terminal sequences. The amplified 606 bp product and  
15 pCON6 were digested with SacII and MluI and the 4094 bp fragment of the vector as well as the PCR product was ligated together to create pWAP.6. The  $\beta$ -galactosidase ( $\beta$ -gal) gene of E. coli was cloned into the resulting vector pWAP.6 (4687 bp): pWAP.6 as well as pBAGN were digested  
20 with BamHI and the linearised vector fragment as well as the 3072 bp  $\beta$ -gal fragment of pBAGN were ligated together. The resulting plasmid was pWAPBAG (Fig. 10), which is a ProCon vector in which the 3' 445 bp's containing the WAP-NRE, as well as the 5' 143 bp's of the HGH coding sequence,  
25 were inserted in place of the U3 region in the 3'LTR.

The control of the  $\beta$ -galactosidase gene expression by promoters inserted into the polylinker has been validated by infection studies using the constructed MMTV and WAP retroviral vectors to infect various cells.

30 To produce retroviral vector particles, the MMTV and WAP ProCon vectors have been transfected into the packaging cell line GP+E86 (Markowitz et al., J. Virol. 62:1120-1124 (1988)). After selection for neomycin resistance, which is encoded by the vector, stable populations and clones of

recombinant ProCon virus producing cells were obtained. Virus containing supernatant from these populations was used to infect a mouse mammary cell line EF43 (Gunzburg et al., *Carcinogenesis* 9:1849-1856 (1988)) as well as a mouse  
5 fibroblast cell line (Jainchill et al., *J. Virol* 4:549-553 (1969)). Four days after infection, the target cells were lysed and quantitative  $\beta$ -galactosidase assay revealed no expression in either cell type infected by the WAP carrying ProCon vectors and good expression in both cell types from  
10 the MMTV carrying ProCon vector (Fig. 13). This result is in accordance with the WAP promoter only functioning *in vivo* during late pregnancy and lactation and not in most simple *in vitro* mammary cell culture systems as represented by the EF43 cells. To investigate whether the WAP carrying  
15 ProCon vectors would be active in a complex primary mammary derived cell culture system, primary organoids from 8-10 day pregnant mice (Fig. 14) or from mammary tumors (Fig. 16) were taken into culture and infected with the supernatant from the same stably transfected population of  
20 transfected cell lines. Both ProCon vectors carrying the WAP and the MMTV promoter fragments were active in these primary cells (Fig. 14) and mammary tumor derived cells (Fig. 16) as demonstrated by  $\beta$ -galactosidase activity.

To investigate whether the WAP and MMTV carrying  
25 ProCon vectors were active *in vivo* and whether the expression of  $\beta$ -galactosidase was limited to the mammary gland *in vivo*, recombinant ProCon virus containing medium was injected *in situ* into the mammary glands or skin of  
8-10 day pregnant mice. Five days later, the mice were  
30 sacrificed, cell extracts prepared and a  $\beta$ -galactosidase assay performed. Both the WAP and MMTV fragment carrying ProCon vectors were expressed only in the pregnant mammary gland and not in the skin (cf M and S in Fig. 15). Thus *in vivo* the regulatory elements from both promoters limit  
35 expression to the mammary gland whereas *in vitro* the

regulatory elements from the WAP promoter retain their strict tissue specificity but those of MMTV do not.

These ProCon vectors carrying tissues specific promoters and regulatory elements will be useful for directing the expression of therapeutic genes to predefined cell types, tissues and organs. Potential therapeutic genes include melittin, which has anti-HIV and anti-tumor effects, and genes which prime cells for death including the thymidine kinase, guanine phosphoribosyltransferase and cytosine deaminase genes, cytochrome P450, as well as genes involved in cell cycle regulation such as SDI/WAF1/CIP-1.

#### EXAMPLE 2

Validation of promoter conversion in cells infected with a ProCon vector that originally carried the MMTV promoter in the 3'LTR:

A ProCon vector carrying the promoter region from mouse mammary tumor virus (MMTV) was transfected into a packaging cell line and the resultant recombinant vector particles used to infect an established human bladder carcinoma cell line (EJ). Infected cell clones were selected in medium carrying the neomycin analog G418 (since the vector carries a neomycin resistance gene driven from an internal SV40 promoter). DNA was prepared from one of the infected clones and nontransfected parental EJ cells and used for Polymerase Chain Reactions (PCR). The PCRs were performed using one of two primers that specifically recognize and bind to MMTV sequences (A, B in Figs. 11 & 12) or the MLV R region (C in Fig. 11) of the LTR together with a primer located within the marker gene (Figs. 11 & 12). Since the marker gene primer is only located downstream of the MMTV (or MLV R region) sequence if promoter conversion has occurred, a positive PCR signal obtained with the MMTV primers in combination with the marker gene

primer is indicative of this. In Fig. 4 the PCR products using primers A, B or C are shown after hybridization to a labelled fragment from the MLV sequence, verifying that all three PCR products are of MLV origin. The size of the fragments verifies that promoter conversion has occurred. Fig. 5 shows the PCR products using primer A or B and hybridized to an MMTV specific probe, again verifying that promoter conversion has occurred.

### EXAMPLE 3

#### 10 Construction of ProCon Vectors for targeted integration:

Using the same BAG vector described in Example 1 above, a retroviral vector can be constructed in which a DNA sequence with homology to a cellular sequence can be inserted into the LTR. The resulting vector can be used to target the integration of either the homologous sequence inserted into the vector or the whole or part of the vectors into the homologous sequence present in the host cell genome.

According to the principle set forth above, a fragment of the thymidine kinase (tk) gene of herpes simplex virus (HSV) has been inserted into the polylinker region of the modified BAG vector (tk mutant in Fig. 17, Saller, R.M., "Design von Locus- und Gewebespezifischen Retroviralen Vektoren Ruer Eine *In Vivo* Gentherapie, Doctoral thesis, Ludwigs-Maximilians University Munich, Germany, (1994)).

A cell line has also been established that has no functional copy of the mammalian tk gene and instead carries one copy of the HSV-tk gene (Saller, R.M., "Design von Locus- und Gewebespezifischen Retroviralen Vektoren Ruer Eine *In Vivo* Gentherapie, Doctoral thesis, Ludwigs-Maximilians University Munich, Germany, (1994)). This cell line has been infected with the tk carrying BAG

The above examples have illustrated the principles and consequences of the promoter conversion vectors provided by the present invention.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

CLAIMS

We claim:

1. A retroviral vector which is capable of undergoing promoter conversion comprising a 5' long terminal repeat region of the structure U3-R-U5; one or more sequences selected from coding and non-coding sequences; and a 3' long terminal repeat region comprising a completely or partially deleted U3 region wherein said deleted U3 region is replaced by a polylinker sequence, followed by the R and U5 region.
2. The retroviral vector according to Claim 1 wherein the 3' long terminal repeat region comprises a completely deleted U3 region.
3. The retroviral vector according to Claim 1, wherein said polylinker sequence comprises at least one unique restriction site.
4. The retroviral vector according to Claim 3, wherein said polylinker sequence comprises at least one insertion of a heterologous DNA fragment.
5. The retroviral vector according to Claim 4, wherein said heterologous DNA fragment is selected from one or more elements of the group consisting of regulatory elements and promoters.
6. The retroviral vector according to Claim 5, wherein said regulatory elements and promoters are target cell specific in their expression.

7. The retroviral vector according to Claim 6, wherein said target cell specific regulatory elements and promoters are selected from one or more elements of the group consisting of Whey Acidic Protein specific regulatory elements and promoters, Mouse Mammary Tumor Virus specific regulatory elements and promoters,  $\beta$ -lactoglobulin and casein specific regulatory elements and promoters, pancreas specific regulatory elements and promoters, lymphocyte specific regulatory elements and promoters and Mouse Mammary Tumor Virus specific regulatory elements and promoters conferring responsiveness to glucocorticoid hormones or directing expression to the mammary gland.
8. The retroviral vector according to Claim 5, wherein said regulatory elements and promoters regulate the expression of at least one of the coding sequences of said retroviral vector.
9. The retroviral vector according to Claim 1, wherein each long terminal repeat region is selected from at least one element of the group consisting of a long terminal repeat region of Murine Leukaemia Virus, Mouse Mammary Tumor Virus, Murine Sarcoma Virus, Simian Immunodeficiency Virus, Human Immunodeficiency Virus, Human T Cell Leukaemia Virus, Feline Immunodeficiency Virus, Feline Leukaemia Virus, Bovine Leukaemia Virus, and Mason-Pfizer-Monkey Virus.
10. The retroviral vector according to Claim 1, wherein said retroviral vector is based on a BAG vector.

11. The retroviral vector according to Claim 1, wherein said coding sequence is selected from one or more elements of the group consisting of marker genes, therapeutic genes, antiviral genes, antitumor genes, and cytokine genes.
12. The retroviral vector according to Claim 11, wherein said marker or therapeutic gene is selected from one or more elements of the group consisting of  $\beta$ -galactosidase gene, neomycin gene, Herpes Simplex Virus thymidine kinase gene, puromycin gene, cytosine deaminase gene, hygromycin gene, secreted alkaline phosphatase gene, guanine phosphoribosyl transferase (gpt) gene, alcohol dehydrogenase gene and hypoxanthine phosphoribosyl transferase (HPRT) gene.
13. The retroviral vector according to Claim 1, wherein at least one of said coding sequences for a retroviral protein is altered or at least partially deleted.
14. The retroviral vector according to Claim 1, wherein retroviral sequences involved in integration of retroviruses are altered or at least partially deleted.
15. The retroviral vector according to Claim 4, wherein said heterologous DNA fragment is homologous to one or more cellular sequences or a part thereof.
16. The retroviral vector according to Claim 5, wherein said regulatory elements are regulatable by transacting molecules.

17. A retroviral vector system comprising:  
a retroviral vector which is capable of undergoing  
promoter conversion comprising a 5' long terminal  
repeat region of the structure U3-R-U5; one or more  
sequences selected from coding and non-coding  
sequences; and a 3' long terminal repeat region  
comprising a completely or partially deleted U3 region  
wherein said deleted U3 region is replaced by a  
polylinker comprising regulatory sequences, followed  
by the R and U5 region; and
- a packaging cell line harboring at least one  
retroviral or recombinant retroviral construct coding  
for proteins required for said retroviral vector to be  
packaged.
18. The retroviral vector system according to Claim 17  
wherein the packaging cell line harbors retroviral or  
recombinant retroviral constructs coding for those  
retroviral proteins which are not encoded in said  
retroviral vector.
19. The retroviral vector system according to Claim 17  
wherein the packaging cell line is selected from the  
group consisting of psi-2, psi-Crypt, psi-AM, GP+E-86,  
PA317 and GP+envAM-12.
20. A method for introducing homologous or heterologous  
nucleotide sequences into target human or animal cell  
populations *in vitro* and *in vivo* comprising infecting  
the target cell population with recombinant  
retroviruses produced by the retroviral vector system  
of Claim 17.

21. The method according to Claim 20, wherein the  
nucleotide sequences are selected from one or more  
elements of the group consisting of genes or parts of  
genes encoding for proteins, regulatory sequences and  
promoters.
22. Recombinant retroviral particle obtained by  
transfecting a packaging cell line of a retroviral  
vector system according to Claim 17 with the  
retroviral vector according to Claim 17, and culturing  
the cells under suitable conditions.
23. A retroviral provirus produced by infection of target  
cells with a recombinant retroviral particle according  
to Claim 22 whereby the polylinker in the 3' long  
terminal repeat becomes duplicated during the process  
of reverse transcription in the target cell and  
appears in the 5' long terminal repeat as well as in  
the 3' long terminal repeat of the resulting provirus.
24. mRNA of the retroviral provirus according to Claim 23.
25. RNA of a retroviral vector according to Claim 1.
26. Pharmaceutical composition containing a  
therapeutically effective amount of a recombinant  
retroviral particle according to Claim 22.
27. A method for introducing homologous or heterologous  
nucleotide sequences into target human or animal cell  
populations *in vitro* and *in vivo* comprising infecting  
the target cell population with the recombinant  
retrovirus particle of Claim 22.

## Abstract of the Disclosure

The present invention relates to a retroviral vector  
5 undergoing promoter conversion comprising a 5'LTR region of  
the structure U3-R-U5; one or more sequences selected from  
coding and non-coding sequences; and a 3'LTR region  
comprising a completely or partially deleted U3 region  
wherein said deleted U3 region is replaced by a polylinker  
10 sequence, followed by the R and U5 region. The retroviral  
vector undergoes promoter conversion and is useful as a  
gene transfer vehicle for targeted gene therapy.

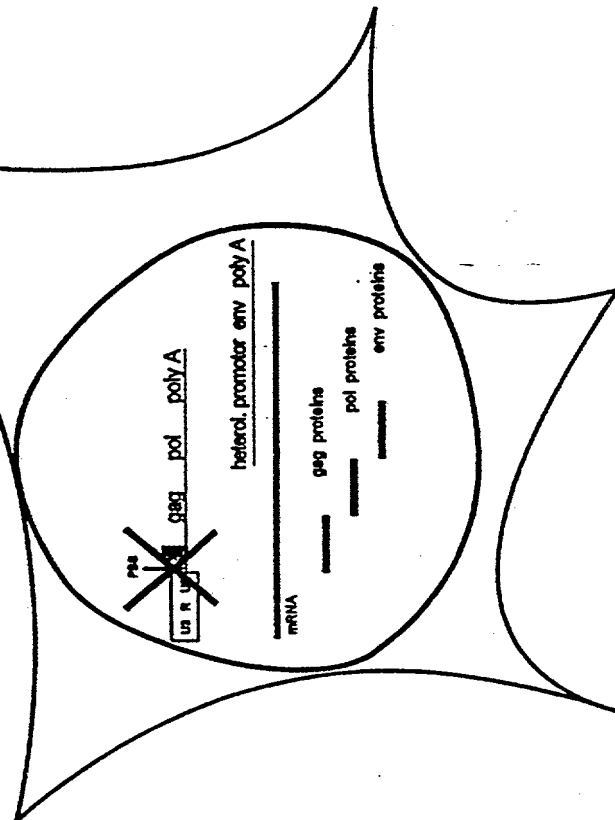
# retroviral Vector

Packaging signal



transfection

packaging cell line

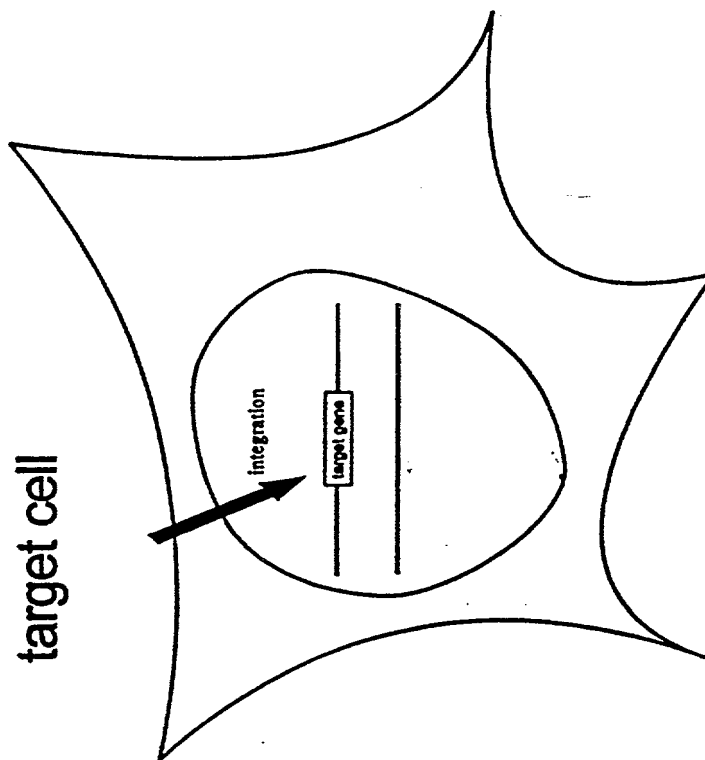


recombinant Virus



infection

target cell



release

Fig. 1

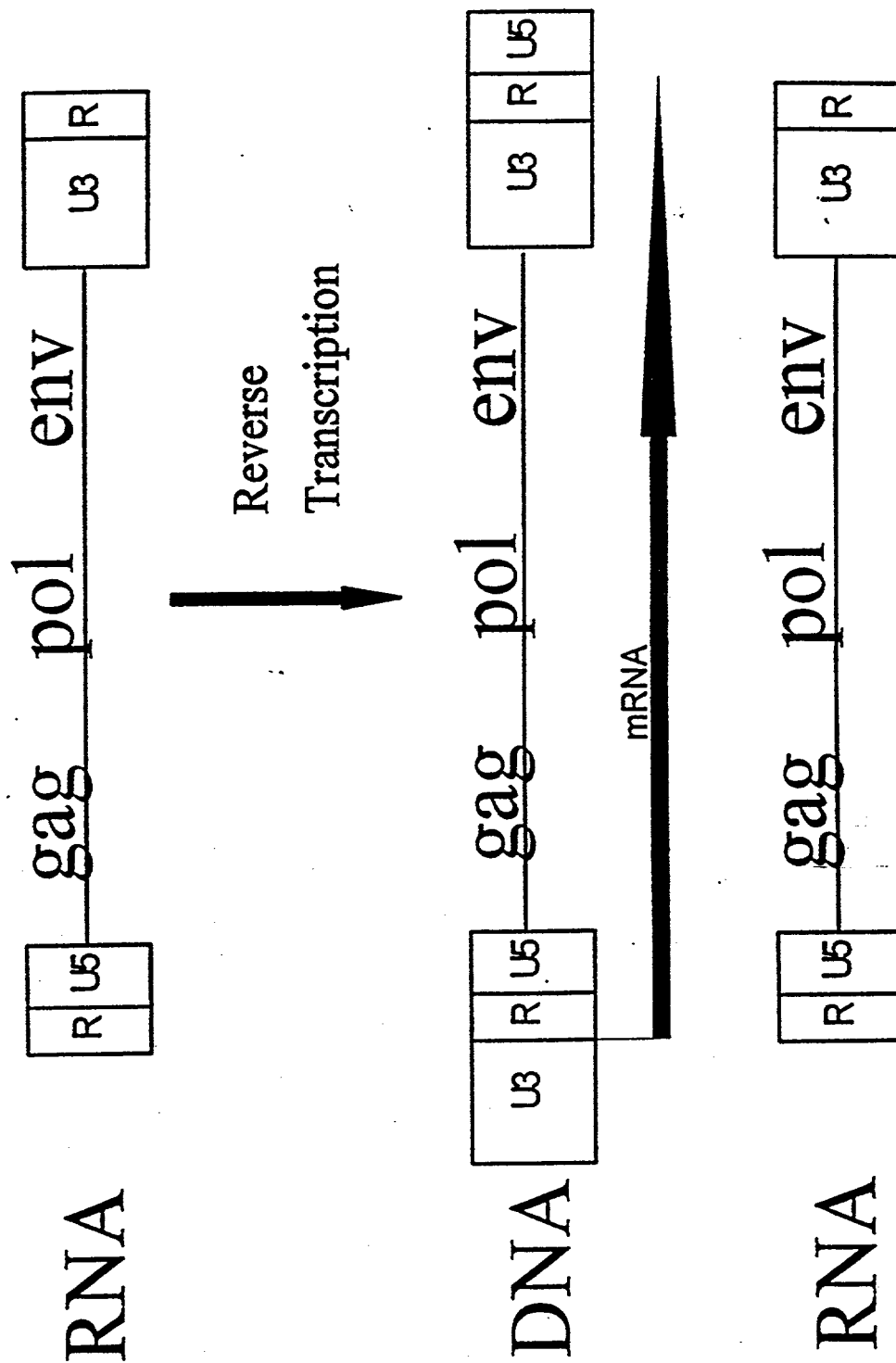


Fig. 2

# Construction of a U3 minus BAG-vector (MLV)

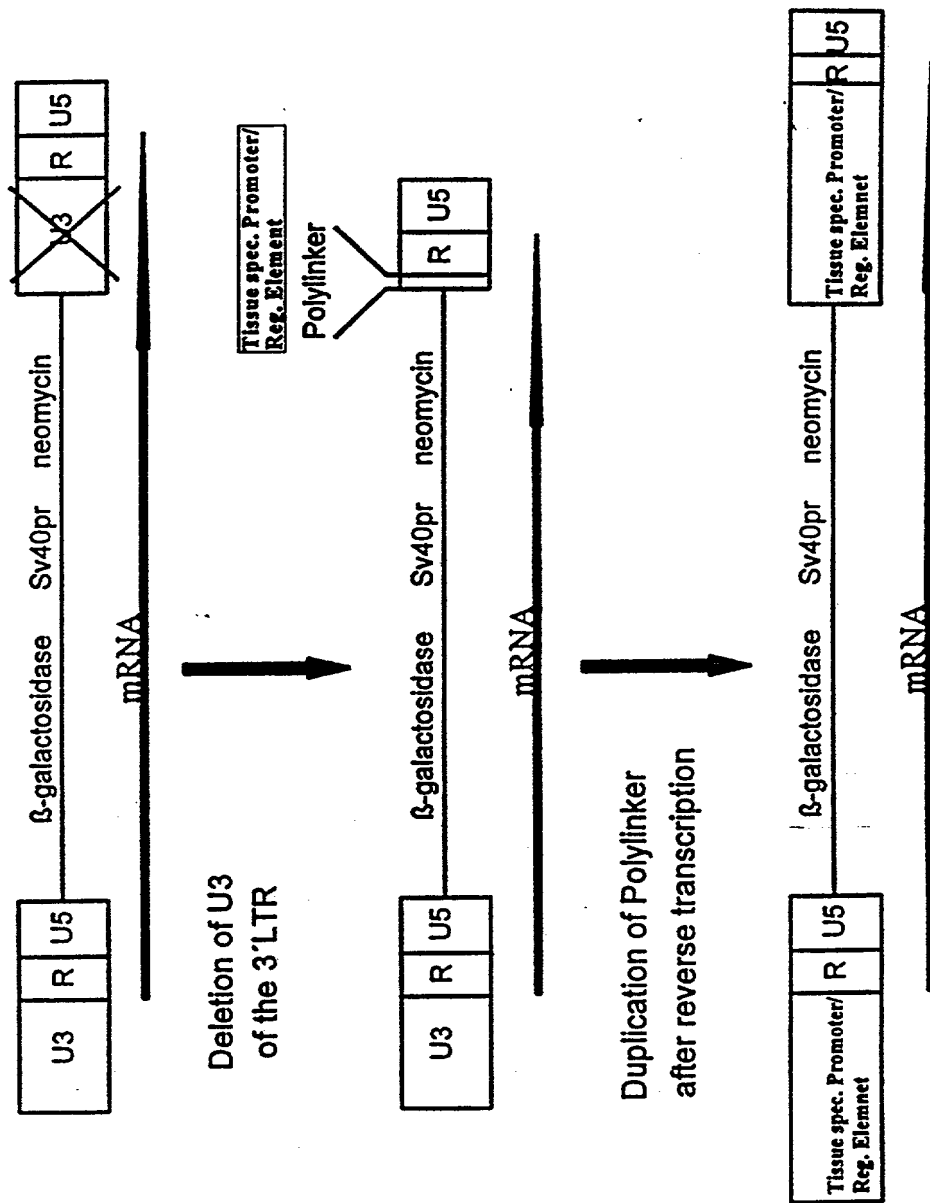


Fig. 3

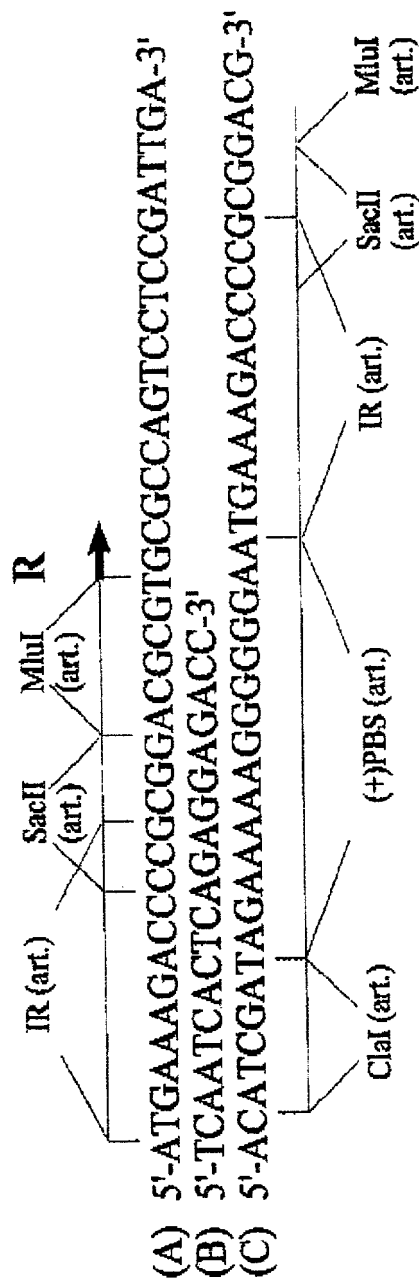


Fig. 4

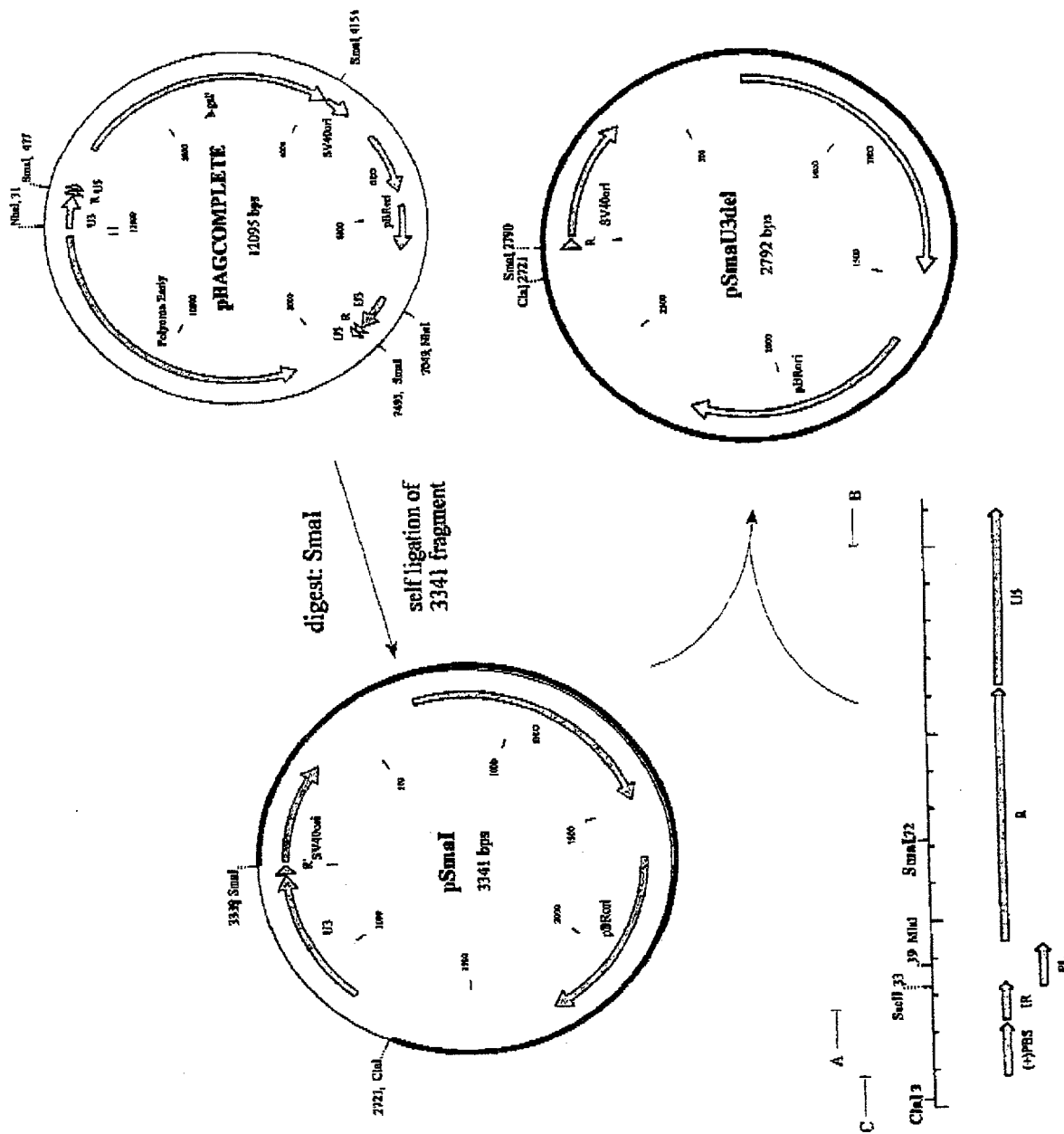


Fig. 5

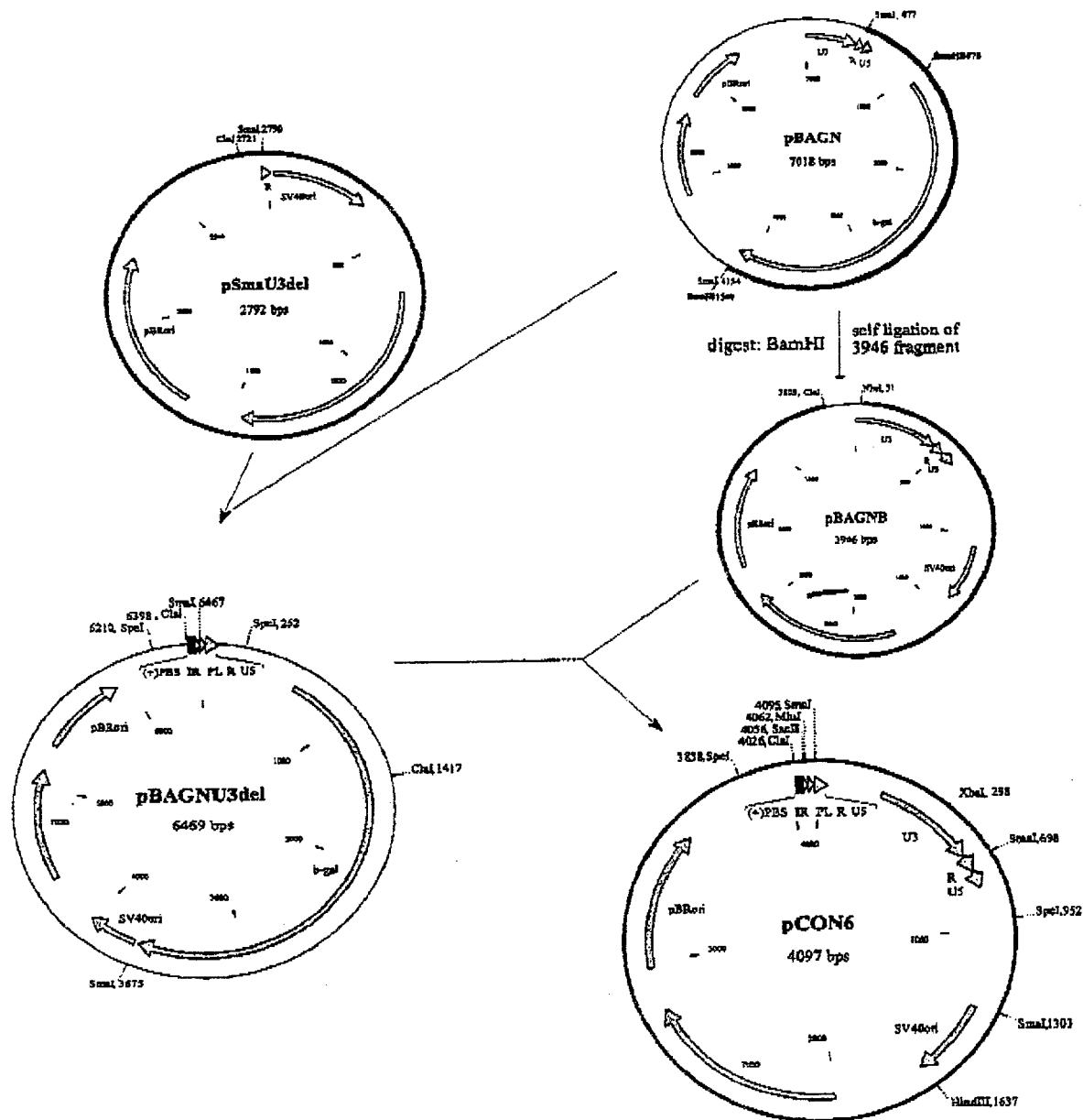


Fig. 6

SacII (art.)

MMTV-U3

(D) 5'-ATACCGCGGAAAA AGGGGAAATGCC-3'

(E) 5'-GCCGGCAGCGGT AAGTTACTCAAAAATCAGC-3'

**MIUI (art.)**

MMTV-U3

Fig. 7

Fig. 8

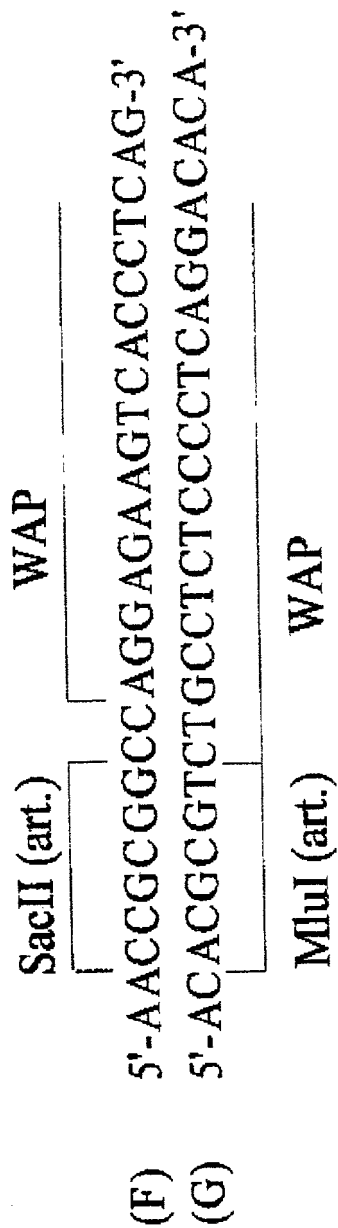


FIG 7

Fig. 9

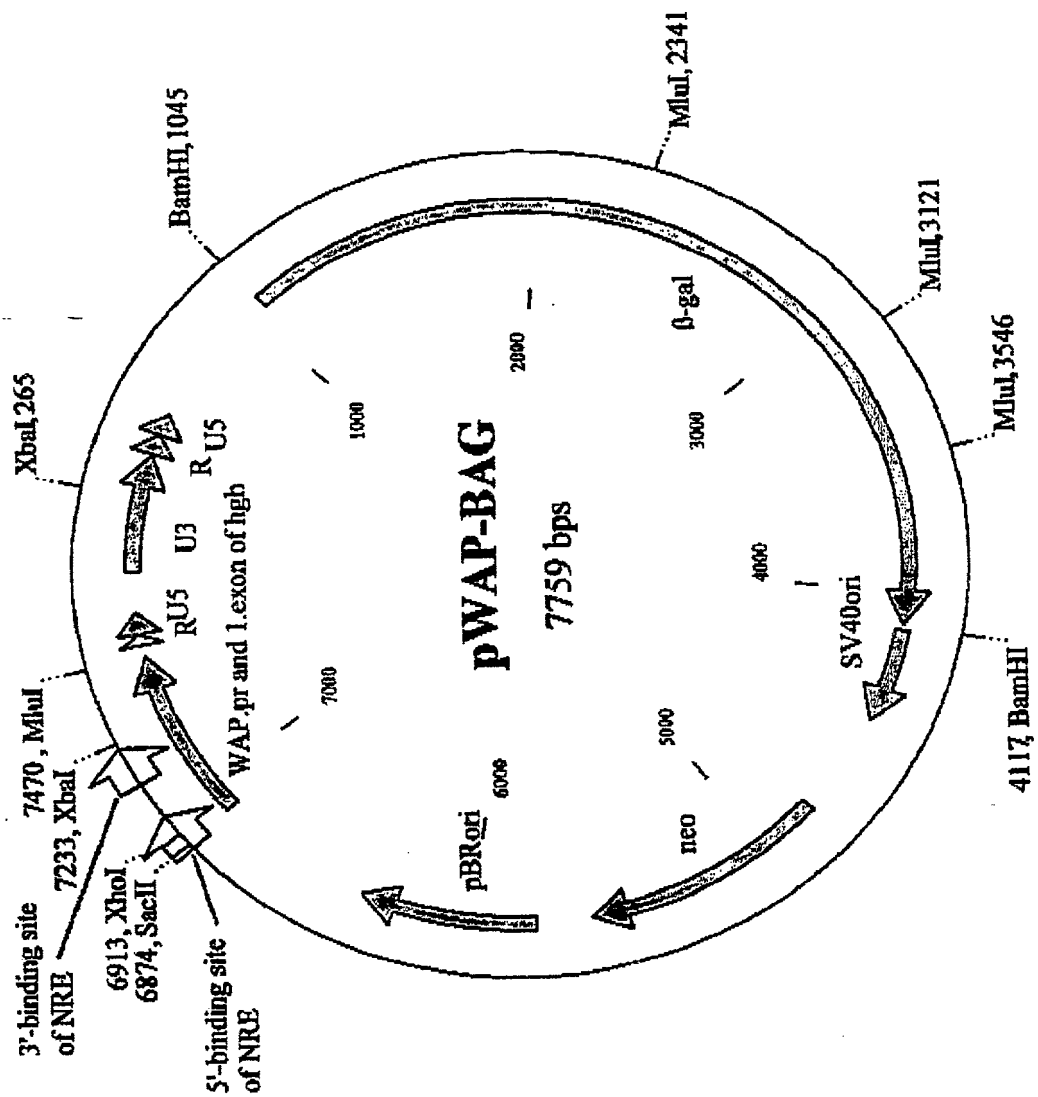


Fig. 10

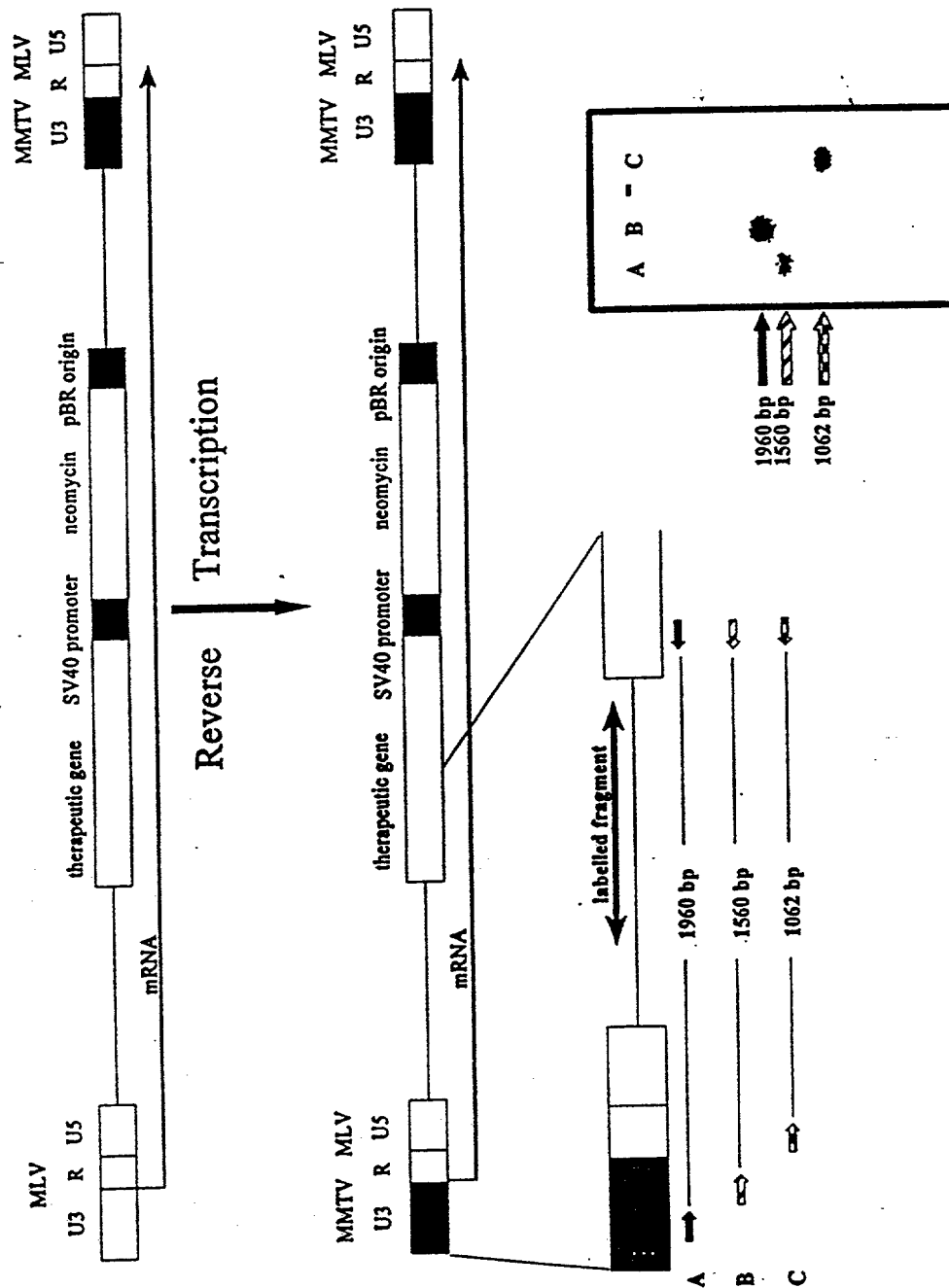


Fig. 11

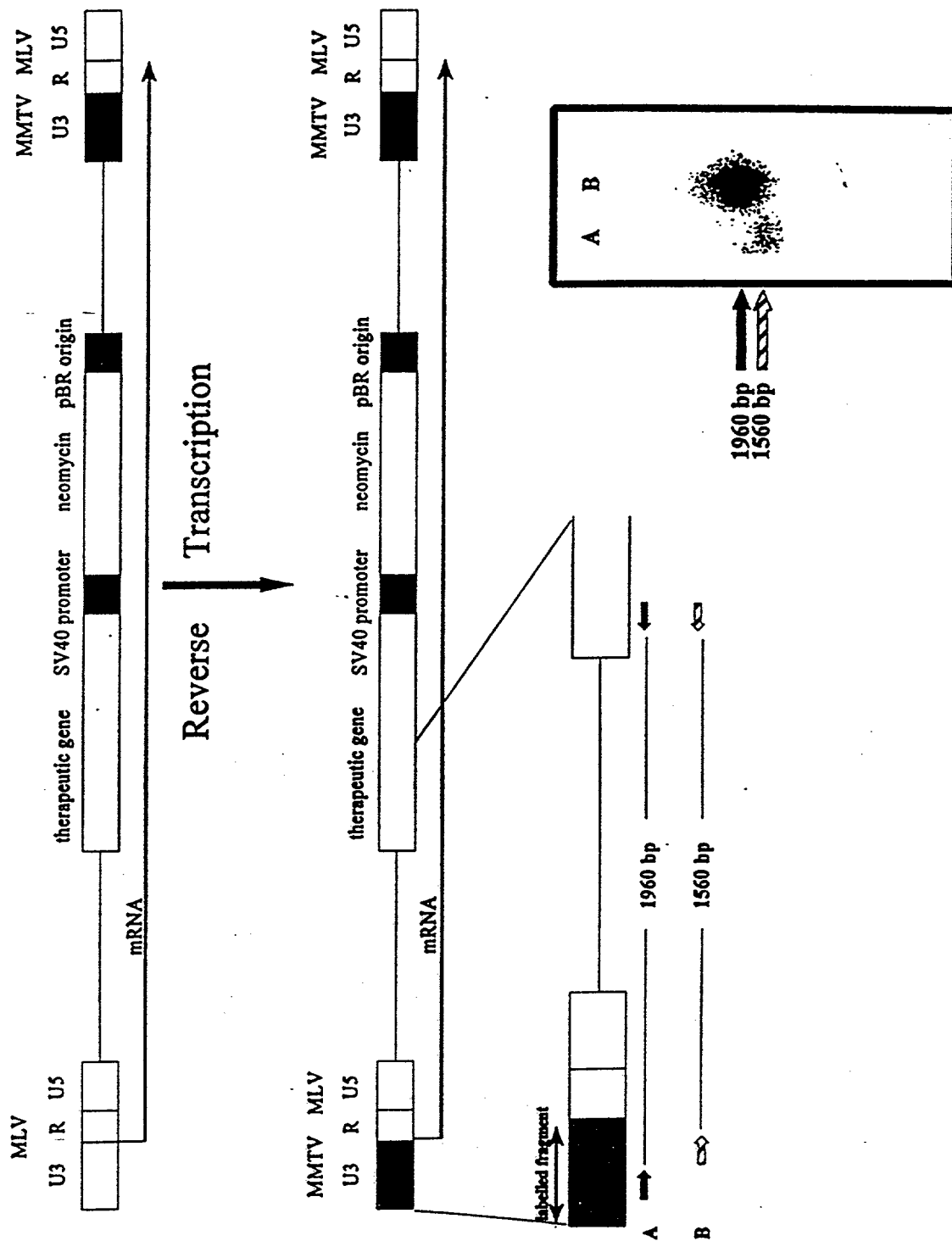


Fig. 12

68220" 2880880

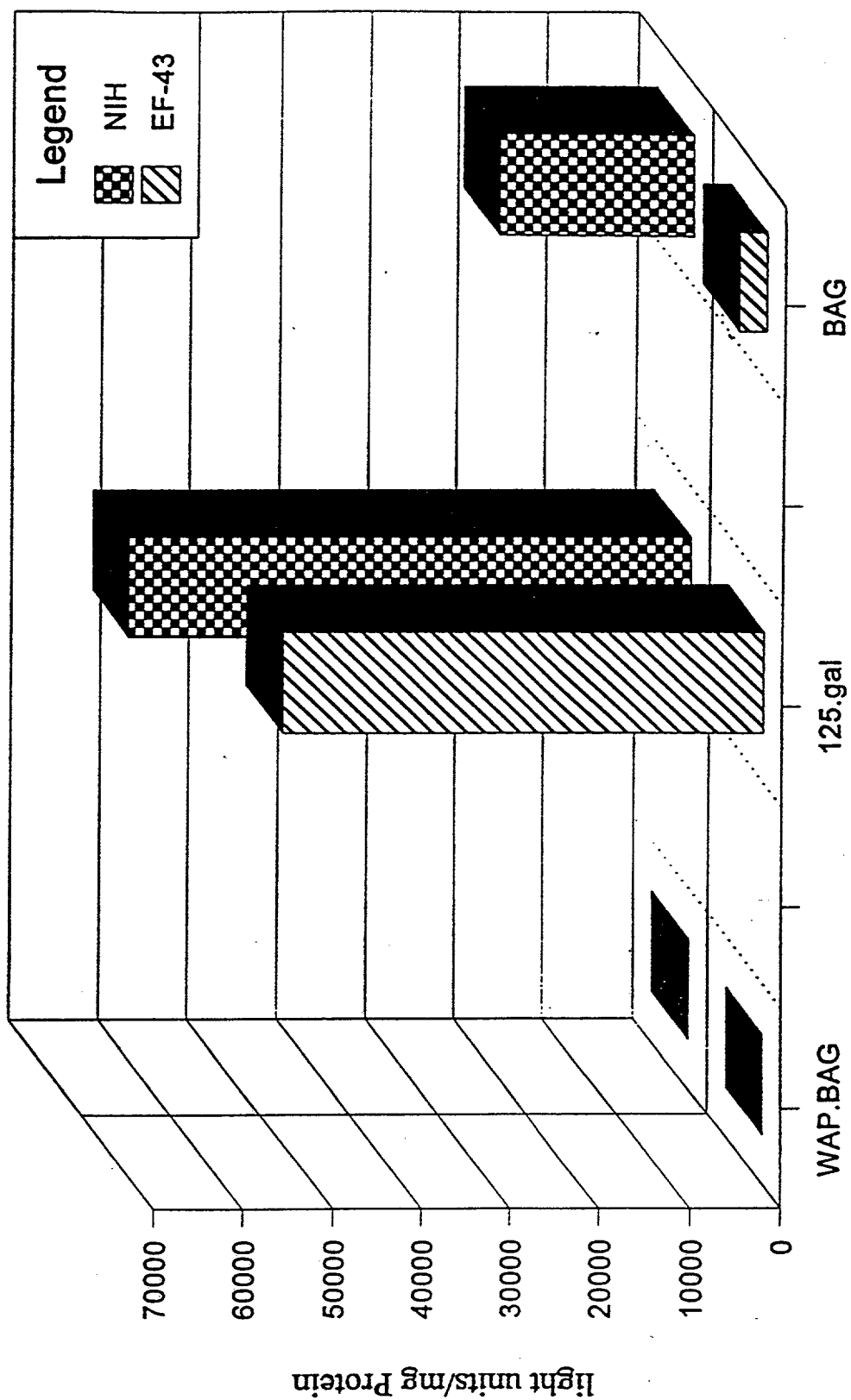


Fig. 13

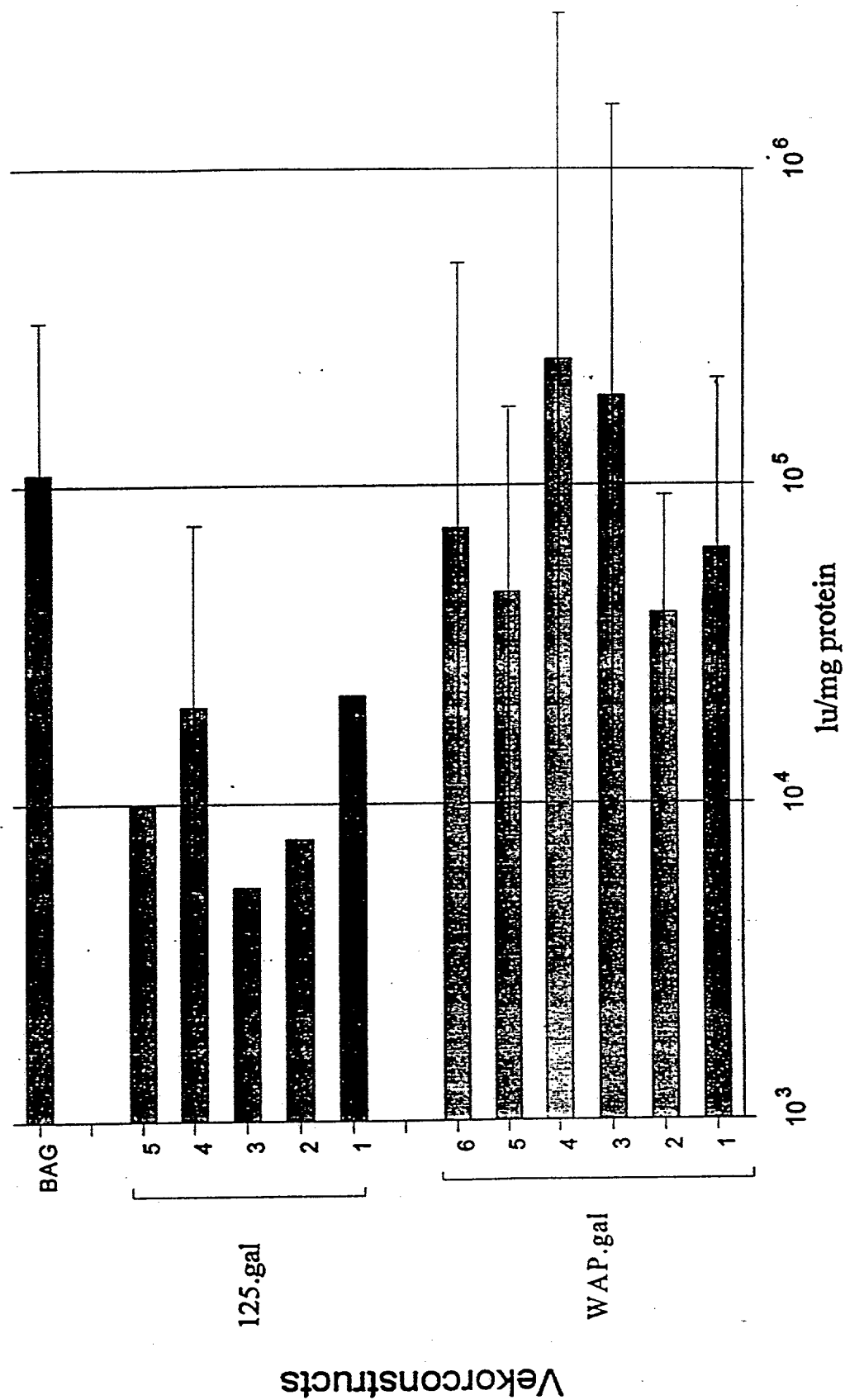


Fig. 14

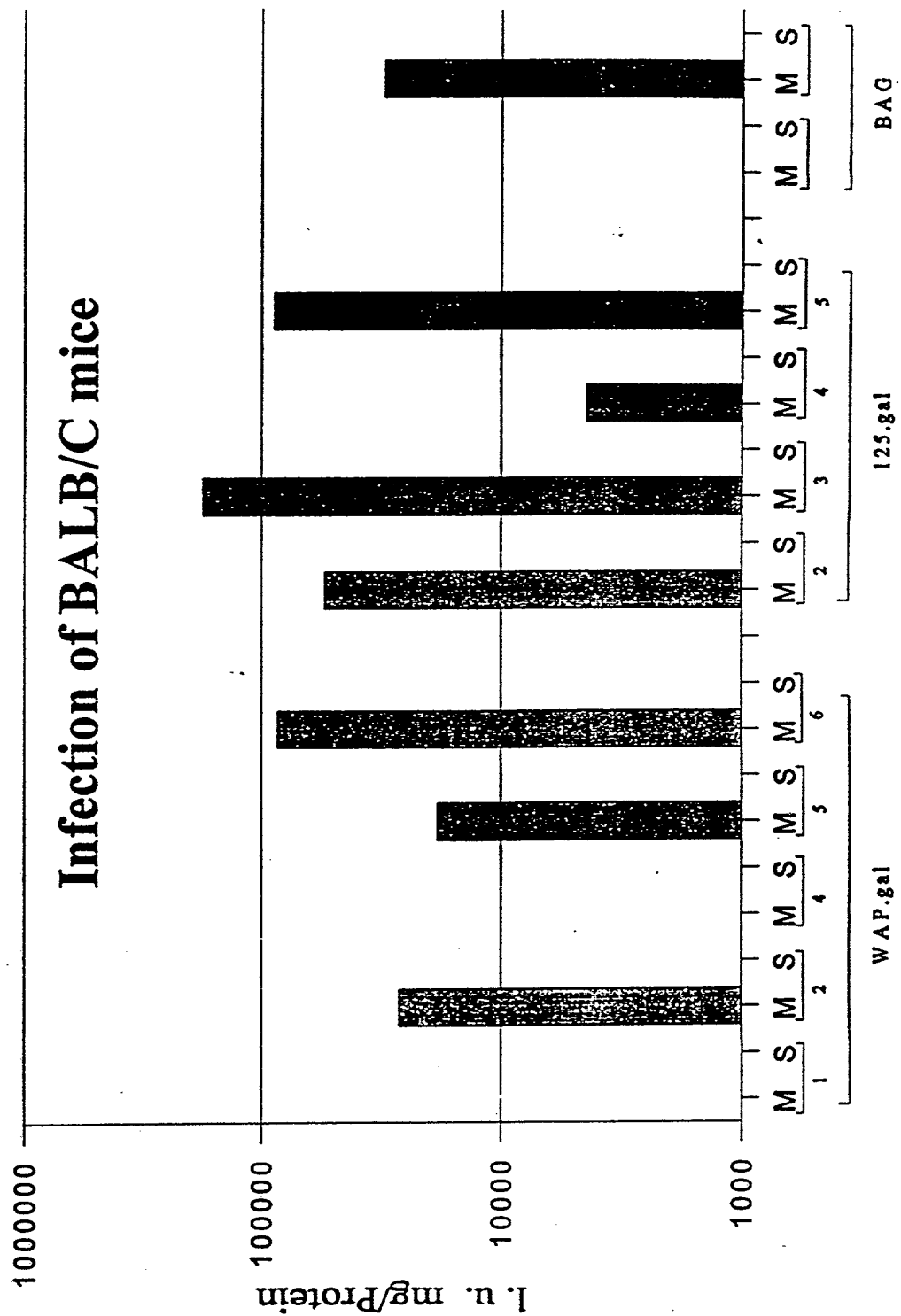


Fig. 15

# **β-gal assay for infected Mouse Mammary Tumour Cells**

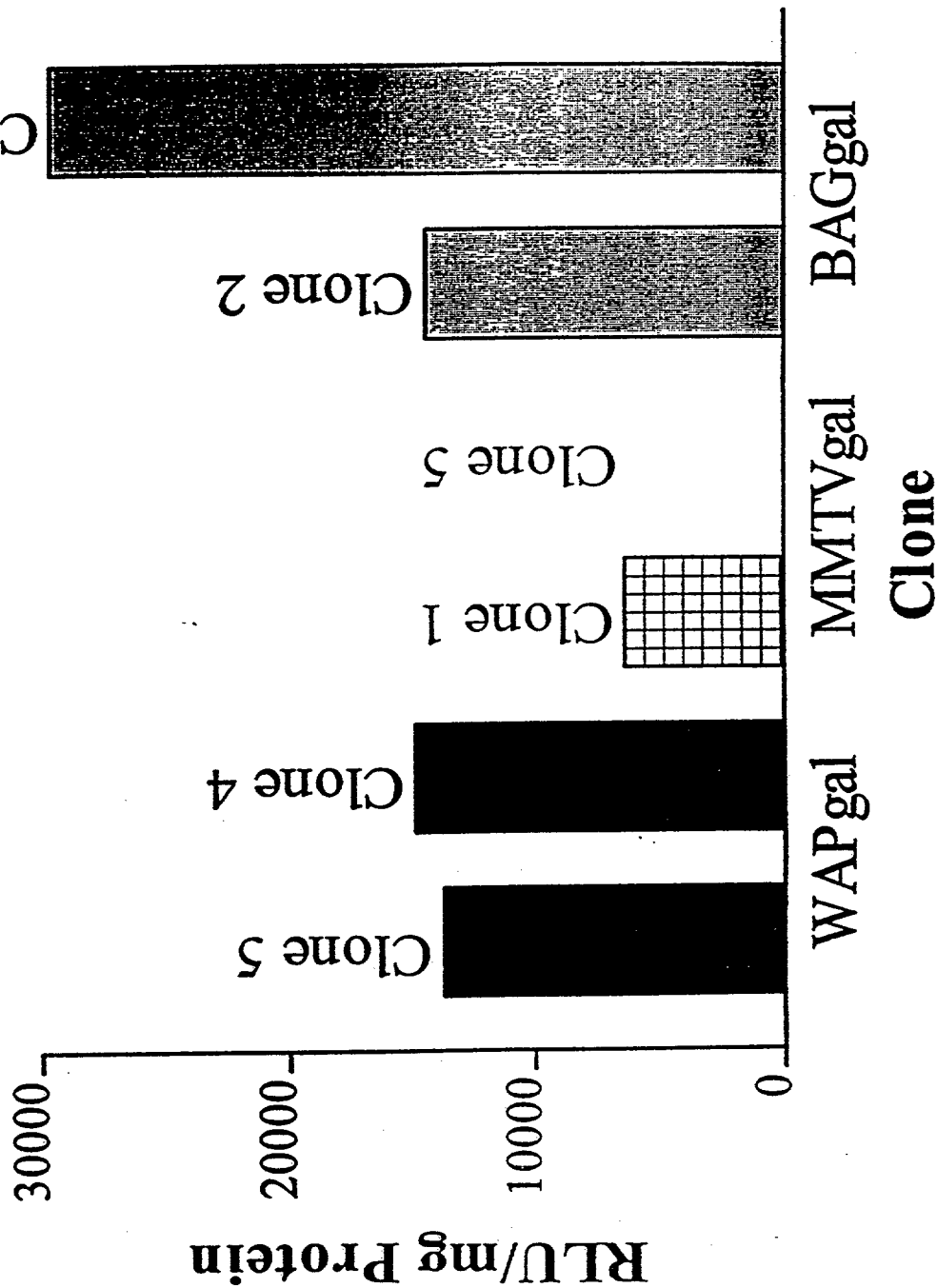


Fig. 16

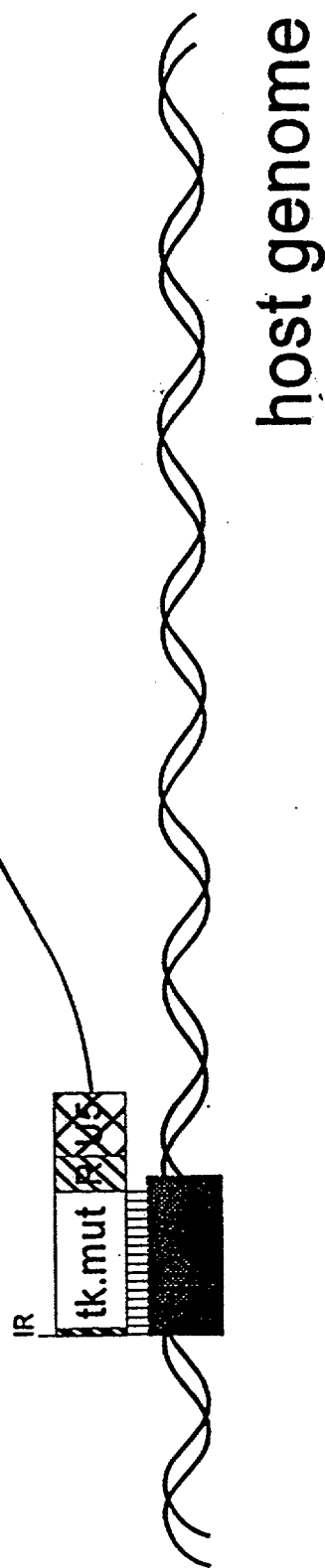


Fig. 17

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Declaration for Patent Application

As a named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name;

I believe I am the original, first and sole inventor (if only one name is listed) or an original, first and joint inventor (if plural names are listed in the signatory page(s) commencing at page 3 hereof) of the subject matter which is claimed and for which a patent is sought on the invention entitled

NON SELF-INACTIVATING, EXPRESSION TARGETED RETROVIRAL

VECTORS

the specification of which (check one)

☒ is attached hereto.

☐ was filed on \_\_\_\_\_ as United States Application

Number or PCT International Application Serial No. \_\_\_\_\_

and was amended on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

<u>Prior Foreign Application(s)</u>			Priority Not Claim
<u>PCT/EP95/03445</u> (Number)	<u>PCT</u> (Country)	<u>                    </u> (Day/Month/Year filed)	[    ]
<u>1017/94</u> (Number)	<u>Danish</u> (Country)	<u>                    </u> (Day/Month/Year filed)	[    ]
<u>                    </u> (Number)	<u>                    </u> (Country)	<u>                    </u> (Day/Month/Year filed)	[    ]

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

                      
(Application Number)

                      
(Filing Date)

                      
(Application Number)

                      
(Filing Date)

0380827 02837

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information known by me to be material to patentability as defined in 37 C.F.R. §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

<u>(Application Serial No.)</u>	<u>(Filing date)</u>	<u>(Status, patented, pending, abandoned)</u>
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<u>(Application Serial No.)</u>	<u>(Filing date)</u>	<u>(Status, patented, pending, abandoned)</u>
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<u>(Application Serial No.)</u>	<u>(Filing date)</u>	<u>(Status, patented, pending, abandoned)</u>
---------------------------------	----------------------	---

<u>(Application Serial No.)</u>	<u>(Filing date)</u>	<u>(Status, patented, pending, abandoned)</u>
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As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

I also hereby grant additional Powers of Attorney to the following attorney(s) and/or agent(s) to file and prosecute an international application under the Patent Cooperation Treaty based upon the above-identified application, including a power to meet all designated office requirements for designated states:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Full name of third joint

inventor, if any \_\_\_\_\_

Inventor's

Signature \_\_\_\_\_ Date \_\_\_\_\_

Residence \_\_\_\_\_  
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